Regenerative medicine in orthopaedic surgery

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ABSTRACT

Regenerative medicine includes the use of technologies aimed at repairing or replacing damaged cells, tissues and organs, in order to restore their structure and function. The clinical indications for the use of regenerative medicine in orthopaedic surgery are degenerative diseases (arthritis, aseptic necrosis, osteochondritis), posttraumatic conditions (non-union) and osteoarticular segmental bone loss. The objective of tissue regeneration in orthopaedic surgery can be achieved with minimally invasive techniques or using open surgery with the application of biological or synthetic scaffolds, autologous mesenchymal stem cells, growth factors or specific surgical techniques and new-generation surgical devices. Three-dimensional bioprinting, the new frontier of tissue engineering, is a promising technology for regenerative medicine in orthopaedic surgery. In the present review, all the different techniques of bone tissue regeneration will be described with the aim of highlighting their evidence-based effectiveness and trying to define their specific role in different indications.

KEYWORDS

Bone defect, mesenchymal stem cell, growth factors, bone graft, bone regeneration, bioprinting.

Introduction

Regenerative medicine includes the development and use of technologies aimed at repairing or replacing damaged cells, tissues and organs, in order to restore their structure and function. In orthopaedic surgery, the main focus of regenerative medicine is bone and cartilage tissue, although it is also applied to muscles and tendons, with the aim of addressing a wide range of musculoskeletal disorders. The objective of tissue regeneration in orthopaedic surgery can be achieved with minimally invasive techniques through percutaneous injections or using open surgery with the application of biological or synthetic scaffolds, autologous mesenchymal stem cells (MSCs), growth factors (GFs) or specific surgical techniques and new-generation surgical devices.

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Over the past five decades, regeneration of segmental bone defects has represented the holy grail of orthopaedic surgery. The first-choice approach was to use an autologous bone graft (free or vascularized), which still today remains the gold standard for bone regeneration. During the 1980s, allogenic and synthetic grafts were introduced, due to the development of bone banking facilities in several Western countries. At the same time, an innovative surgical technique was proposed by Ilizarov, whereby bone tissue regeneration was obtained by means of bone transportation mediated by an external fixator [1,2]. During the nineties, regenerative medicine became popular: in this case, adult stem cells and GFs were used in an attempt to recreate the conditions necessary for bone tissue repair and growth. In the same period, the osteoinductive membrane technique was introduced for segmental bone reconstruction and showed successful outcomes in several challenging clinical situations [3]. Most recently, innovative technologies, non-invasive intramedullary lengthening devices and hexapodalic external fixators have been revolutionary in the field of bone regeneration.

Articular cartilage is avascular and has the limitation of being unable to proliferate and repair mechanical damage. In the case of degenerative or posttraumatic osteochondral defects, the traditional treatment is arthroscopic debridement and microfracture, with the aim of inducing subchondral bone bleeding, with subsequent fibrocartilage proliferation filling the defect.

In larger defects of the articular cartilage, autologous osteochondral grafts retrieved from the femoral condyle or allogenic grafts are commonly used. As an alternative to these traditional techniques, autologous chondrocyte implantation has been introduced; this consists of autologous articular cartilage sampling and reimplantation after *in vitro* growing with or without a scaffold membrane. More recently, the use of synthetic scaffolds, alone or as a carrier for MSCs and GFs, has become a widespread technique[4].

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In the last decade, the integration of 3D technology and bioprinting has led to three-dimensional bioprinting, making it possible to create three-dimensional structures including biological matrix and cells. This technology, whose ultimate objective is to create tissues and organs, offers future perspectives for regenerative medicine.

In the present review, all the regenerative medicine techniques used in bone tissue regeneration will be described to highlight their evidence-based effectiveness, examine their outcomes and complications, and try to define their specific role in different indications.

From the triangular to the diamond concept

Traditionally, the principles of bone restoration and regeneration have been based on three factors: osteogenic cells, osteoinductive GFs and osteoconductive scaffolds. This combination, known as the "triangular concept", is well represented in autologous bone grafting, commonly recognized as the gold standard for bone regeneration. In 2007, Giannoudis *et al*. introduced the "diamond concept", also including mechanical stability and vascularity as determinant factors for creating conditions favorable to bone growth [5]. In addition, the authors speculated on the need for a closed but permeable space able to accommodate the different bone regeneration factors (scaffold, cells and GFs) in a mechanically stable assembly, and thus introduced the concept of the "biological chamber". Certainly, a vascular environment and mechanical stability create the conditions for synergistic action of osteogenesis, osteoinduction and osteoconduction, provided respectively by cells, GFs and scaffolds [6,7].

Autologous bone grafts

As mentioned above, autologous bone grafts (ABGs) are the gold standard of bone substitutes as they have properties of all three processes required for bone regeneration: osteogenesis, provided by living osteocytes and osteoblasts; osteoinduction, by morphogenetic proteins contained in bone matrix; and osteoconduction, through the three-dimensional trabecular scaffold. The main drawbacks to the use of ABGs are donor site morbidity and limited availability. In a 2011 review, minor and major complications were reported in up to 39% and 10% of cases, respectively, after autologous bone graft harvest, and a correlation with the harvest extension was observed[8]. Persistent residual pain, superficial infection, haematoma formation and superficial nerve lesion were considered minor complications, whereas fracture, intestinal herniation, deep infection and gait disturbance due to gluteal muscle insufficiency were considered major complications [8,9]. It takes ABGs of 5–7cc/cm and 6–12 cc/cm to replace diaphyseal and metaphyseal defects of the tibia and femur respectively [10]. The amount of available ABG is limited, with averages of 36 cc and 20 cc from the posterior and anterior iliac crest, respectively, and 12 cc from the proximal tibia metaphysis reported by Dawson *et al*.[11]. For this reason, an alternative source of ABG has been introduced: the reamer-irrigator-aspirator (RIA) technique allows considerable volumes of ABG, i.e., from 25 to 90 cc, to be harvested from the femur medullary canal, this tissue having biological properties comparable or even superior to those of an iliac crest bone graft, and with lower reported donor site morbidity [12,13]. Nevertheless, due to the problem of limited ABG availability, the reconstruction of large bone defects often requires augmentation with allogenic grafts or synthetic bone substitutes [14]. Indeed, allografts and synthetic scaffold augmentation with MSCs and GFs have become popular techniques, all aiming to provide the same properties as ABG.

Scaffolds

The scaffold is a biocompatible three-dimensional structure with osteoconductive properties, able to promote cellular migration and adhesion. An effective scaffold should have the following characteristics: a large surface (microporosity), in order to enhance the interaction between the cells and the matrix; high viscosity, to allow cell adhesion; macroporosity, to allow capillary diffusion during neoangiogenesis as well as cellular migration; structural mechanical properties for strain resistance; it must also be resorbable, in order to facilitate new bone formation [7]. Biological and synthetic scaffolds are available. Allografts and xenografts are biological scaffolds, consisting of decellularized bone which maintains its osteoconductive properties but lacks osteogenic cells. Allografts can be fresh frozen, in which case they will have better mechanical strength and osteoinductive properties, or freeze-dried and irradiated, with lower resistance and functioning matrix proteins. Demineralized bone matrix contains osteoinductive proteins and can be used as an allogenic scaffold or augmentation of bone substitutes. Several different synthetic scaffolds are available on the market, ranging from resorbable biopolymers to hydroxyapatite, tricalcium phosphate and bioglass. Following the advent of three-dimensional printing and additive manufacturing, synthetic custom-made scaffolds can now be produced with case-specific structure and dimensions [7]. The advantage of biological and synthetic scaffolds is the unlimited supply, but the disadvantage is their lack of osteogenic and osteoinductive potential. To overcome this limitation, several authors have augmented scaffolds with autologous MSCs and/or autologous or synthetic GFs, obtaining promising results [15,16].

Mesenchymal stem cells (MSCs)

Human adult MSCs are progenitor cells, present in musculoskeletal tissues in order to maintain their integrity through regeneration in response to injury. Unlike true stem cells, which are able to self-regenerate indefinitely, progenitor cells have a limited capacity for self-renewal. When appropriately stimulated, MSCs can proliferate and migrate, presenting a multilineage potential to differentiate into bone, cartilage, fat, muscle and tendon. MSCs are present in several adult tissues but the most commonly used sources are bone marrow and adipose tissue [17]. The use of MSCs *in vivo* is strictly controlled by regulatory authorities, especially concerning expansion *in*

vitro, which is considered "extensive manipulation", by the drug regulatory bodies both in Europe and the US. Conversely, MSC concentration by centrifugation is considered "minimal manipulation" and this practice is routinely employed directly in operating theatres where bone marrow aspirate concentrate (BMAC) or adipose-derived stromal vascular fraction (SVF) can be obtained. Although BMAC is preferred for the harvesting of MSCs in orthopaedics, a higher concentration of connective tissue progenitors has been found in fat tissue than in bone marrow [17]. However, while some authors postulated that BMAC and SVF have similar osteogenic potential, other studies demonstrated a lower action of fat-derived cells and absence of osteoblastic progenitor surface markers [18-20]. MSC concentration in bone marrow has also been seen to be dependent on age, gender and associated systemic diseases. A lower concentration was found in females and a decrease with age progression was observed. The importance of the BMAC harvesting technique in increasing the concentration of progenitor cells has been underlined, it being recommended to limit the volume of each aspiration to no more than 2-5 mL, in order to avoid dilution with peripheral blood. The concentration of progenitor cells can be enhanced 5-fold using centrifugation and 20-fold through forced perfusion in absorbable scaffolds. MSCs are employed in different ways in several orthopaedic diseases, e.g., in percutaneous injections in degenerative osteoarthritis and non-unions and in open procedures for bone defect regeneration, where they are used in association with scaffolds and osteoinductive GFs [15,21,22].

Growth factors (GFs)

The first phase in the natural process of fracture repair is the hematoma formation with the beginning of the coagulation cascade. The protagonists of this phase are macrophages and platelets. The former clean up necrotic debris, while the latter degranulate releasing several cytokines and GFs, including proinflammatory cytokines like interleukins 1, 6, 8, 10 and 12, tumor necrosis factor α TNFα, activated protein C, monocyte chemoattractant protein, macrophage colony stimulating factors, receptor activator of nuclear factor kappa-B ligand RANKL and osteoprogenin [2]. At the same time, an important role in bone repair is played by metalloproteinases and vascular endothelial growth factor (VEGF). However, the most effective cytokines in stimulating proliferation and differentiation of progenitor cells into osteoblastic lineage are platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor beta (TGFβ), a large category of cytokines including bone morphogenetic proteins (BMPs) 2, 4, 6 and 7 [23]. Platelet-rich plasma (PRP) is a platelet concentrate obtained from the patient's peripheral blood. Platelets are small (2μm diameter) cytoplasmatic fragments of megakaryocytes containing different granules (α, δ, λ). As mentioned, when activated by the coagulation cascade, platelets degranulate releasing cytokines, as well as (in particular from α-granules) several mediators active in bone regeneration, including VEGF, PDGF, IGF, FGF and TGFβ. These GFs stimulate neoangiogenesis and have a chemotactic action towards progenitors of osteoblastic lineage, stimulating their proliferation and differentiation [24]. As PRP contains high concentrations of GFs, it has become widely used in several orthopaedic conditions including percutaneous injections in degenerative arthritis and tendon enthesopathy. At the same time, PRP has been shown to be effective as augmentation of allogenic or synthetic grafts. In animal models, comparable results in restoring bone defects were observed between ABGs and calcium phosphate augmented with PRP and BMAC [25]. The combination of a scaffold (osteoconductive), GFs (osteoinductive) and MSCs (osteogenic) has been widely used in orthopaedic clinical applications with the aim of recreating the biological conditions of autogenous bone grafts and the triad necessary for bone regeneration [11,12]. Percutaneous infiltrative or minimally invasive techniques are commonly used in arthritis, non-unions, avascular necrosis and osteochondritis, while open surgery is reserved for reconstruction of cavitary or segmental bone defects [24].

After introducing the concept of osteoinduction in 1965, Marshall Urist identified the initiating agent of this process as a protein contained in bone matrix called bone morphogenetic protein (BMP) [26,27]. Since then, through molecular biology, a family of BMPs has been identified that includes more than sixteen proteins contained in bone matrix. All these proteins belong to the transforming growth factors β superfamily TGFβ, a group of GFs playing an important role in tissue repair. Some of these proteins showed the capacity to signal for chemotaxis, proliferation and differentiation of MSCs into osteoblasts and to promote enchondral bone formation [28]. Among all the BMPs, the most potent in osteoinduction were found to be BMP-2 and BMP-7 and, after almost three decades of research, recombinant BMP-2 and 7 were introduced into clinical practice in the late nineties. It was found that the efficacy of BMPs depended on their concentration. The natural delivery system is highly effective because BMPs are retained in the bone matrix which acts as a reservoir. To enhance prolonged delivery of BMPs and increase their in-site concentration, appropriate carriers able to retain and progressively release BMPs in the implant site were studied. For clinical applications, recombinant BMP-2 and 7 were integrated in a type-1 bovine collagen carrier [28]. Another option to obtain local controlled release of BMPs is gene therapy, which can be performed through transfection of host cells with genes encoding for BMPs by means of *in vivo* or *in vitro* transduction [29]. However, concerns regarding viral vectors and possible immune reactions have limited the clinical application of this technique. Several studies demonstrated the efficacy of BMP-2 and 7 to improve union rate in long bone fractures and non-unions and spinal arthrodesis [30-33]. Despite these successful preliminary results, at present, BMPs are not yet part of common clinical practice, probably also due to the high cost and difficulties in obtaining controlled in-site release.

Distraction osteogenesis

Bone regeneration through segmental transportation is known as "distraction osteogenesis". The concept was introduced in the early 1950s by Gavril Abramovich Ilizarov in

the Soviet Union but it did not become popular in the Western world until the early '80s [1,2]. His brilliant idea, based on segmental bone transport through a circular external fixator, was successfully applied to the treatment of fractures, septic and aseptic non-unions, deformity correction and reconstruction of critical bone defects. Excellent results were reported with this method, with a mean bone union rate >90% and external fixation index of around 1.5 months/cm [34]. The circular external fixators principle has since been widely applied to monolateral and hybrid fixators. In particular, it rapidly became the gold standard treatment of septic non-unions of long bones, allowing critical-size defect restoration after extensive debridement of necrotic and infected bone stumps [34]. At the end of bone transport, docking site non-union can be observed. To overcome this complication, augmentation of the docking site with autologous bone graft or conversion of the external fixator to a plate, or intramedullary nail fixation, has been recommended [34]. Another option is the acute limb shortening and re-lengthening technique, which is useful in reconstructing a combined bone and soft tissue defect. In this case, the bone stumps are placed in contact through limb shortening while a proximal metaphyseal osteotomy is performed and bone transport by an external fixator is initiated to restore the limb length [35]. Despite the successful results, the drawbacks of distraction osteogenesis using the external fixator method include the long treatment duration, patient discomfort, the need for compliance, and frequent pin site infection. For this reason, the introduction of intramedullary lengthening devices has been considered a revolutionary improvement of distraction osteogenesis. Initially conceived as mechanically activated devices, transforming rotatory movements into linear elongation, motorised non-invasive intramedullary nail distractors were introduced in the'90s [36]. These revolutionary devices were based on telescopic sliding of the nail components activated electronically through radiofrequency transmission or by the use of an electromagnetic field [36,37]. After osteotomy, a controlled progressive elongation is achieved by means of interaction between the external remote controller and the magnet inside the nail [38]. Non-invasive lengthening nails are currently available for femur and tibia and they are successfully employed for distraction osteogenesis in the treatment of limb length discrepancy and in deformity correction in congenital or posttraumatic conditions or oncologic reconstructions [39,40]. In particular, the association of intramedullary lengthening nail with bridging plate fixation allows the reconstruction of posttraumatic bone defects [41]. Recently, a new non-invasive intramedullary nail specifically designed for bone transportation has been introduced, with a view to replacing the combined (nail + plate) technique.

Osteoinductive membrane

The induced-membrane (IM) technique, first described by Masquelet in 1986, is a two-stage procedure used for intercalary segmental bone regeneration [42]. The first surgical stage consists of careful debridement of the bone defect followed by the application of a polymethylmethacrylate cement spacer and internal (plate or nail) or external fixation. The spacer has a dual effect: the first is mechanical, preventing fibrous tissue invasion of the bone defect; the second is biological, promoting the induction of a surrounding foreign-body granulation membrane (synovium-like epithelium) with high osteoinductive potential [43]. In the second surgical stage, the IM is carefully divided, the spacer is removed, and the remaining defect is filled with ABG retrieved from the iliac crest or by means of RIA. Depending on the size of the defect, the autologous graft may be augmented with allogenic grafts or bone substitutes taking care to not exceed a 3:1 ratio [3]. With the use of antibiotic-loaded cement spacers, the IM technique has been found to be effective even in irradiated or infected surgical fields. Studies on animal models showed the IM to be a vascularised collagen-based membrane containing macrophages and lymphocytes as well as osteoclasts and osteoprogenitor cells. Moreover, the secretion by the IM of several growth factors (VEGF, TGF-β-1, BMP-2, etc.) with both neoangiogenic and osteoinductive potential was observed [44-46].

The timing of the second stage procedure is considered a determinant of the success of the technique, as the highest concentration of GF secretion was seen to occur 4–8 weeks after spacer implantation [43]. Nevertheless, failures of the technique have been described, being found to be related to the defect extension, autograft-bone substitute ratio, stability of the fixation, and timing of second stage [3,47]. With the aim of identifying predictive biomarkers of osteoinductive potential of IM, serum levels of metalloproteases and insulin-like growth factor-1 have been identified as a promising tool [45,46]. Surgical tips to improve the outcome and success rate of the IM technique have also been described: extensive debridement of the bone defect is critical, with removal of all necrotic tissue especially in cases of septic non-union; the medullary canal should be opened, reamed and irrigated; the aim is to obtain two healthy bleeding bone stumps; a stable fixation is recommended, using external fixation in septic conditions and internal fixation in aseptic bone defects; when positioning the cement spacer, care should be taken to insert the cement inside the medullary canal (up to 2cm) and to overlap the cortical bone of the stumps; adequate soft tissue coverage with well vascularized tissues should be achieved; the autologous bone graft must be used in the second stage, and possibly augmented with allogenic grafts or bone substitutes not exceeding a 3:1 ratio; multiple microbiological samples are recommended in both stages [3,47]. Bone grafts can be augmented with osteoprogenitor cells from bone marrow aspirate or osteoinductive GFs, autologous from PRP or commercially available BMPs. The IM technique is indicated and commonly employed to reconstruct posttraumatic bone defects or segmental bone loss due to infection, non-union, tumors or congenital pseudoarthrosis [3]. In the two largest series reported, the IM technique was used to reconstruct posttraumatic bone defects with an overall union rate higher than 90% [48,49].

Vascularized bone grafts

Vascularized bone grafts (VBGs) have been widely used for intercalary reconstructions of long bones since they integrate all the properties required for bone regeneration: osteogenesis, osteoinduction and osteoconduction. VBGs have their

own biomechanical properties; they heal by primary union and have the capacity to undergo hypertrophy in response to load, with the potential to replace even large bone defects. They can be used as pedicled flaps in certain anatomical sites, such as the wrist (carpal non-unions and osteonecrosis), the leg (fibular flap for tibial defects), the spine (rib for posterior spinal fusion), and the knee (medial femoral condyle for distal femur non-union), or as free vascularized flaps [50]. The vascular pedicle has been seen to remain patent even several weeks after anastomosis, providing a segment of viable bone vascularised by both intraosseous perforating vessels and periosteal supply. The presence of living osteocytes and blood supply allows the vascularised graft to maintain the original mechanical strength and to heal, achieving early union with host bone through an accelerated remodelling process (as compared with the creeping substitution of non-vascularised grafts) that includes osteoblastic activity, and osteoid and new bone formation. In addition, spontaneous fracture healing and hypertrophy in response to mechanical stress are commonly observed with viable segmental bone grafts [51]. Several free vascularised bone flaps have been described including the fibula (diaphysis or proximal epiphysis), iliac crest, medial femoral condyle, medial femoral trochlea, scapula, rib and metatarsal physis [50]. VBGs can be harvested as simple bone grafts or as composite grafts including a skin island, a muscle or both (skin and muscle) [50]. Since early reports in the seventies, free vascularised fibula has been widely used for reconstruction of intercalary bone defects in different clinical scenarios [52]. Besides the extensive oncological resections, the indication for its use in non-oncological conditions is usually failure of previous attempts with more traditional techniques (bone transport, osteoinductive membrane technique) or the presence of an unfavourable biological environment, due to local or systemic factors [53]. Free vascularized fibula should be considered in irradiated fields, in septic defects or previously infected areas, in the presence of highly fibrotic or scarred tissue with scarce soft tissue coverage, or in cases of vascular injury where the transected vessel can be used for pedicle anastomosis [53]. Due to the capability to revascularize necrotic bone, free vascularized flaps are commonly used in osteonecrosis of the femoral head, carpal scaphoid and talus [54]. Successful results have been reported using free vascularized fibula as salvage procedure of non-union of fractures of irradiated bone [55].

Decision making on which free vascularized flap to use in a specific clinical situation is based on the site and size of the bone defect as well as consideration of soft tissue and vascular conditions in order to assess whether a composite flap is required and to ascertain vascular inflow and outflow options [50].

Three-dimensional bioprinting

Three-dimensional bioprinting was born from the integration of 3D printing technology and tissue engineering.

Rather in the way bioprinters were derived from traditional inkjet printers in the 1980s, 3D bioprinters use the operating principles of 3D printing to create tissues and organs, layer after layer through the deposition of cells and natural or synthetic polymers, called bioinks [56].

Bioink development is challenging due to the need to take into account and integrate both the biological properties required for cell growth and the structural prerequisites of the printing process [57]. Some bioink formulations make use of hydrogels to encapsulate cells in a matrix that has properties similar to those of the extracellular matrix [58].

Biomimetics and self-assembly are the main principles of bioprinting: printed structures must be similar to the living tissues and the cellular self-assembly mechanisms should allow the transition from the initial printed state to the final complex structure to occur without external intervention [59].

Spheroids (clusters of cells) are deposited on a substrate where they mature into tissues. Due to adhesion molecules, spheroids self-assemble into cell cultures that mimic the processes of embryogenesis, morphogenesis and organogenesis [56].

Currently, the most common 3D bioprinting technologies are inkjet, laser and extrusion based [54]. Inkjet bioprinters use different technologies to deposit ink droplets in a similar manner to traditional desktop inkjet printers. Laser bioprinter technology employs laser energy to release cells from a donor site to the substrate located below the donor site. Extrusion bioprinters use the same technology applied to some of the most common and affordable 3D printers. A nozzle is moved over the substratus on the xy plane and it deposits the bioink by means of mechanical or pneumatic extrusion. The bioink is then cross linked [56].

To obtain the three-dimensional characteristics of the tissue, the printing process can be supported by a scaffold. A scaffold allows the creation of a mechanically resistant extracellular matrix. Tissue volume and structure are easily controlled. The scaffold must be biodegradable and biocompatible and the most commonly used materials are polymers and bioceramics [60]. Most research into bone tissue bioprinting focuses on the use of scaffolds, to achieve initial structural integrity. Various combinations of polymers and bioceramics have been developed to create scaffolds with bone-like characteristics [60]. Bioprinting, in the absence of a scaffold, enhances cell-cell and cell-extracellular matrix interactions. However, scaffold-free techniques need to address the problem of poor resistance to compressive forces.

Finally, bioprinting includes an additional step: it is essential to print endothelial cells to allow bone tissue, especially in the case of large segments, to receive the necessary vascularization and nourishment [61].

Bioprinting represents the most promising future perspective for regenerative medicine in the field of orthopaedic surgery. Although the use of tissue engineering in clinical practice is still limited by technical and regulatory hurdles, bioprinting is expected to become an essential tool to improve the clinical outcomes of orthopaedic surgery patients.

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